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Infrared laser triggered release of bioactive compounds from single hard shell microcapsules

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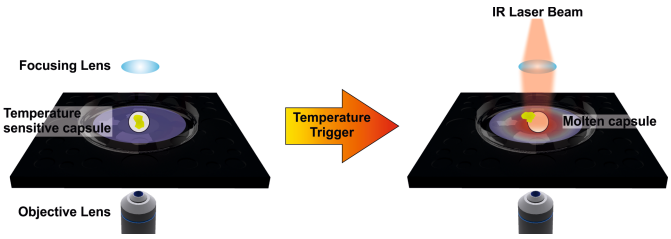
Micro composites are commonly characterized in bulk. Here we study the temperature triggered release of a bioactive compound from single isolated microcapsules. We monitor the release process in real-time using a novel thermal microscopy method combining laser-induced heating and fluorescence imaging.

Microencapsulation of functional compounds (for example flavors, proteins or vitamins) can be of great value for the use in food, cosmetic or pharmaceutical industry[1-6]. Encapsulation is a way to increase shelf life, improve stability and control the release of the encapsulated active compounds[7].

For the analysis and characterization of microcapsule products, techniques such as Differential Scanning Calorimetry[8], Nuclear Magnetic Resonance[9], thermogravimetric analysis[10] or viscosity-measurements are applied. Furthermore, bright field or electron microscopy are used to determine particle size, shape and surface characteristics[11]. These techniques report sample averaged properties, without accounting for heterogeneity of the samples. To characterize the melting of temperature sensitive, single microcapsules with high spatio-temporal resolution we implement a new tool to trigger and visualize the release of active compounds. The technique combines infrared laser heating and high speed microscopy[12] (Scheme 1). In comparison to other thermal microscopy methods which evenly heat the whole sample[13] this method heats microcapsules in the focal spot of the laser only. To demonstrate the method, we produced a model system by entrapping a biological active in a temperature sensitive, hard shell microcapsule.

There are various encapsulation techniques available to entrap actives like spray-drying, freeze-drying, rapid expansion of supercritical solutions (RESS), coacervation, particle from gas saturated solutions (PGSS), biopolymerization and emulsification[20, 21]. Drawbacks, however, are increased costs and the additional complexity of handling the encapsulation process. For the preparation of our model system we have chosen the hot melt dispersion technique[14, 15], as it is cost effective, easily executable and can be performed in lab scale as well as in industrial scale. The hard fat Witepsol W31, which has a melting temperature close to the human body temperature, was used as the shell material. As core material Enhanced Yellow Fluorescent Protein (eYFP) was selected. eYFP is derived from the Green Fluorescent Protein (GFP)[16]. eYFP and other fluorescent proteins are widely used as reporters in cells or animals[18, 19]. It has a molecular weight of 27 kDa and is mainly composed of β-sheets arranged in a barrel like shape. The barrel embraces the chromophore and shields it from the environment[17]. The melting temperature of the protein is 78 °C, similar to the melting temperature of GFP[16]. The protein emits fluorescent light at a maximum of 527 nm when excited at 514 nm. Oxidative stress, denaturants or degradation can damage the fluorophore and lead to a decay of its fluorescence. This allows the implementation of its fluorescence as a marker for native protein encapsulation. In this work we show that the technique can be used to determine the melting temperature of single microcapsules and to image the melting process and compound release.



Scheme 1 Schematic representation of the experimental setup to trigger the release of eYFP from hard shell microcapsules. IR laser pulses are used to instantaneously heat the sample in the focal spot thereby melting the shell of the capsule.

We prepared the capsules using the hot melt dispersion technique. This technique utilizes a water in oil in water (W/O/W) emulsion which was rapidly cooled down to solidify the Witepsol W 31 shell material to form stable capsules. The production process led to a reproducible, narrow particle size distribution with an average d_{50} of 130 ± 14 µm and a span of 1.4 ± 0.1 (Figure 1A). The used preparation parameters resulted in different capsule types which were categorized as mono-or polynuclear in morphology (Figure 1B)[22]. A representative, tomographic view of a mononuclear core-shell capsule is shown in Figure 1C. The capsule was 185 µm in diameter with a core diameter of 110 µm. Localization of the fluorescence signal in the core region showed encapsulation of eYFP. Assuming a spherical shape for both the capsule and the core, a core: capsule volume ratio of 1:5 was calculated.

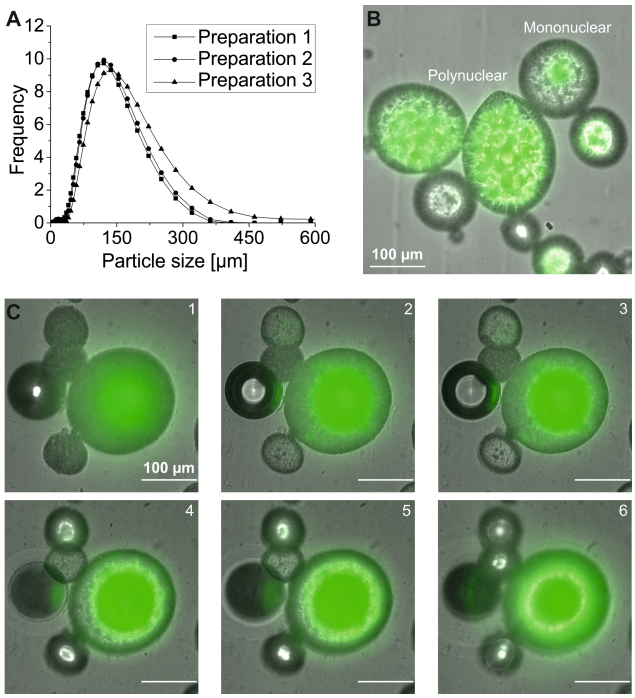


Figure 1. Particle size distribution (PSD) and morphology of the capsules produced by hot melt dispersion. (A) PSD of different capsule preparations determined by laser diffraction. (B) Capsules show polynuclear and mononuclear morphologies. (C) Tomographic view of a mononuclear capsule. Each slice has layer thickness of 37.5 µm. The capsule measured 185 µm in diameter with a core of diameter of 110 µm.

To demonstrate the stability of the micro release system and its ability to entrap eYFP in its native state at specified storage conditions, 25 mg of the capsules were added to Dulbecco's Phosphate-Buffered Saline (DPBS) and stored at -20 °C, 4 °C and room temperature (RT) over a time period of 7 weeks, respectively. At different time points, the fluorescence intensity of the buffers was determined to detect leaked proteins (Figure 2). The sample stored at RT showed significant leakage of the protein after 12 days. However, the shelf life of the micro release system can be increased by storing it at cooled (4°C) or frozen (-20°C) conditions.

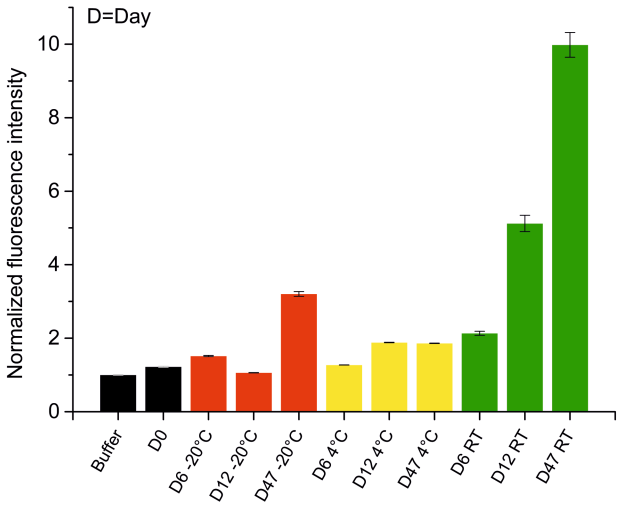


Figure 2. Protein release from capsules at different storage conditions. The capsules were stored at 3 different temperatures (-20 °C, 4 °C and RT) for 7 weeks. Samples of the buffers were taken at the indicated time points and the fluorescence was measured (normalized to buffer). Error bars represent the standard deviation.

We then used the thermal microscopy technique to study the temperature triggered release of eYFP from individual capsules. The capsules were melted in DPBS by infrared laser heating at 2200 nm (see ESI) with the laser focused to heat up a volume of 0.20 mm^3. The melting process of the microcapsule is then imaged by fluorescence microscopy. The laser power was calibrated to heat the sample by using the temperature dependent fluorescence of Rhodamine B (see ESI). Typical heating profiles are shown in Figure S1. The dimensions of the heating spot were selected larger in size compared to the average microcapsule diameter to heat every microcapsule uniformly. Thereby, local temperature gradients across the capsule that could affect the release properties were avoided. However, small laser foci are desirable to selectively heat preselected single microcapsules in the sample (Figure S1). This is a great advantage in comparison to methods where the entire sample chamber is heated such as hot-stage microscopy[13]. Using such methods, all microcapsules loaded to the sample chamber melt and release the bioactive during a single temperature scan. However, as high resolution microscopy is required to monitor the release, only a very few microcapsules can be analyzed and subsequently an exchange of the entire sample is required.

The technique can be operated in two different modes. A fast temperature increase to instantaneously trigger the release of the capsule or successive temperature increases to precisely determine its melting temperature. In the first mode the release is triggered by modulating the laser power output waveform to achieve a single increase in temperature from RT up to the melting temperature (Figure 3A, red curve). The time course of melting a mononuclear capsule is shown in supplemental Video S1 (ESI). A region of interest (ROI) analysis of the supplemental Video S1 is shown in Figure 3. ROI's at cardinal and ordinal positions around the capsule were defined to track the fluorescence intensity during the experiment. After 14 seconds which corresponds to a temperature increase of 10°C above RT the shell breaks at a single spot in the upper left part of the capsule and the liquid core, which contains eYFP, leaked into the surrounding solution. This lead to a major increase in fluorescence of the surrounding ROI's N, W and NW. The fluorescence dissipates over time returning to its initial value (Figure 3). The rapid heating mode allows to trigger and monitor the release of an entrapped bioactive from single microcapsules within a

few seconds. This additionally reduces the time to characterize the release properties in comparison to hot-stage microscopy which usually operates with heating rates up to 10 °C per minute[13].

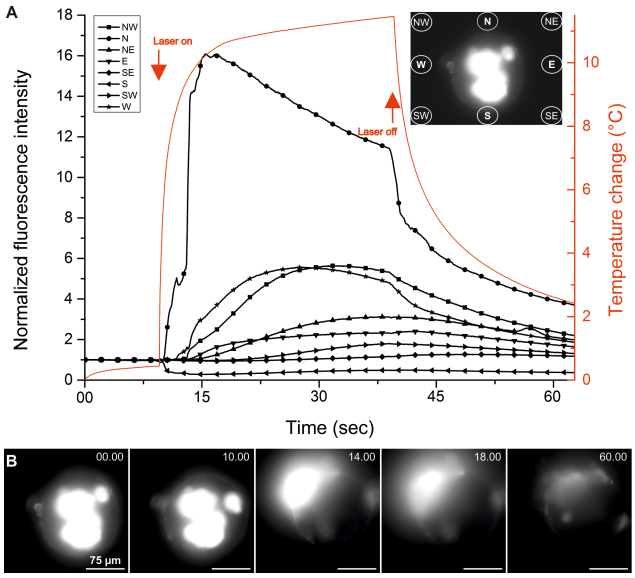


Figure 3. Temperature induced release of a mononuclear capsule. (A) Fluorescence intensity analysis during capsule release. The left axis shows the normalized fluorescence intensity of the shown ROI's (inset) over the duration of the experiment. Cardinal and ordinal directions were used to name the ROI's. The right axis (shown in red) shows the laser induced change in temperature during the experiment. (B) Fluorescence images taken during the heating experiment. 14 seconds after the start of the experiment, corresponding to a temperature increase of 10 °C, the melting temperature of the capsule was reached and eYFP was released resulting in a fluorescence increase in the surrounding regions which dissipates over time.

Secondly, the thermal microscopy technique can be used to slowly increase the temperature to precisely determine the melting temperature of the capsules shell. The laser power output waveform was modulated to create 25 individual heating steps each of 1 °C with 10 s of constant laser power between each step, thus heating the sample up to 50 °C (Figure 4). In the initial heating phase the detected fluorescence intensity around the particle was unchanged showing that the integrity of the shell is not compromised. At 35 °C a slight increase in the fluorescence intensity of ROI N due to a displacement of the capsule was detected. At 36 °C (Figure 4) the capsule breaks at a single spot and the liquid core leaked into the surrounding buffer (see supplemental Video S2 at time 01:40 min). This was accompanied by an increase in the fluorescence intensity of the surrounding buffer, in particular for ROI's E, S and SE (Figure 4A). The regions W and SW are on the far side of the release and showed only a minor increase in the fluorescence intensity. The eYFP protein that diffused into the buffer led to an increase in the normalized fluorescence intensity which returned to its initial value. After the laser is turned off, the sample cooled down to RT which resulted in a rapid solidification of the molten fat. The particle thereby formed amorphous structures and lost its spherical shape (Figure 4B). The melting temperature of 36 °C at which the core was released into the buffer corresponds well with the melting temperature of 35-37 °C reported for the pure fat Witepsol W31.

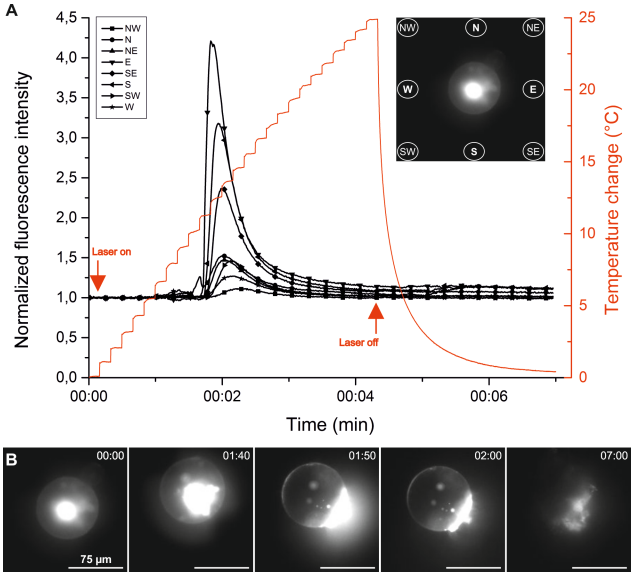


Figure 4. Melting and release characteristics of a mononuclear capsule. A capsule with a diameter of 84 µm was melted. The melting temperature was measured to be 36 °C. (A) Fluorescence intensity analysis of the recorded video. Cardinal and ordinal directions were used to name the ROI's. The right axis (shown in red) represents the laser induced change in temperature during the experiment. (B) Fluorescence images taken during the heating experiment. At time 01:40 min, corresponding to a temperature of 36 °C, the melting temperature of the shell was reached. The encapsulated eYFP solution was released resulting in a fluorescence increase in the surrounding regions which dissipates over time.

Conclusions

The model release system presented here was prepared using the hot melt dispersion technique. The capsules are easily prepared and their properties can be tuned by altering parameters like stirring speed or the selection of the shell material. Our results emphasize that hot melt dispersion is an easily executable and scalable emulsification technique that can be used to prepare hard shell microcapsules for the temperature controlled, instantaneous release of bioactive compounds like proteins or peptides for therapeutic delivery. Furthermore, it can be extended to encapsulate active food ingredients like probiotics. We demonstrate a novel thermal microscopy method for single capsule characterization of temperature triggered release systems. The technique was used to trigger and monitor the release of an encapsulated biological active from a model micro release system. The integrated heating laser can be tuned to rapidly trigger the release of the liquid core or to probe the thermal melting curve with centigrade precision by a stepwise temperature increase. The release and the melting temperature of individual microcapsules can be analyzed and correlated to bulk properties studying size-dependent effects. Future development of the technique such as continuous sample feeding will allow to use the described method in a high throughput fashion.

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